TITLE PAGE

REQUIREMENT FOR MULTIPLE ASSAYS TO DEFINE THE PLASMINOGEN ACTIVATORS IN CONDITIONED MEDIUM FROM CULTURED CELLS

Marian L. Lewis[1], Kevin L. Damron[1]

Grant H. Barlow[2], and Dennis R. Morrison[3]

[3] NASA - Johnson Space Center, Houston, Texas.
Medical Sciences Division, Biomedical Laboratories Branch.
[1] KRUG International, Technology Life Sciences Division, Houston, Texas.
[2] Michael Reese Research Foundation, Chicago, Illinois.

Correspondence:

Marian L. Lewis, Ph.D.
KRUG International
Technology Life Sciences Division
1290 Hercules Drive, Suite 120
Houston, Texas, USA 77058

(NASA-CR-185103) REQUIREMENT FOR MULTIPLE ASSAYS TO DEFINE THE PLASMINOGEN ACTIVATORS IN CONDITIONED MEDIUM FROM CULTURED CELLS (Krug International) 31 p

N89-71116

Unclas
00/51 0213166

KEYWORDS

Plasminogen activator (PA)

Fibrin plate lysis (FPL)

Tissue plasminogen activator (t-PA)

Micro-clot lysis assay (MCLA)

Amidolytic assay (S-2444)

Single chain urokinase-type plasminogen activator (scu-PA)

Urokinase (UK)

SUMMARY

Plasminogen activator (PA) in serum free conditioned medium (CM) from electrophoretically separated subpopulations of human kidney cells was assayed by fibrin plate lysis (FPL), an amidolytic assay (S-2444), a tissue plasminogen activator (t-PA) enzyme linked immunosorbent assay (ELISA), and a micro-clot lysis assay (MCLA). Some of the subpopulations tested by S-2444 and MCLA showed very similar activity; however, the FPL values were much higher. The S-2444 and MCLA did not measure single chain urokinase-type plasminogen activator (scu-PA) which may have been activated in the course of the FPL assay. Preincubation of CM with plasmin and subsequent test by S-2444 showed that samples with negligible activity in the direct assay without plasmin increased to 20-60 IU/ml. The standard FPL assay does not define the contributions to fibrinolysis of either scu-PA converted to active urokinase in the presence of plasmin, or the active form of t-PA. Also, the FPL is less sensitive to plasmin inhibitor found in some CM samples in which the MCLA values were 2-4 times lower than FPL values. The S-2444 and MCLA plasmin activated assays can measure the amount of scu-PA in the sample. The MCLA measures active urokinase plus some, but not all, of the scu-PA in the unactivated samples. For comparison of PA production by separated cell subpopulations, we have shown that a battery of assays must be employed to define the several expressions of the activators.

INTRODUCTION

Since Williams (1) first described urokinase (UK) and the vascular activator was named by Aoki (2), the plasminogen activator (PA) system from a molecular standpoint has become more complex. What at first appeared to be single well defined proteins has now been shown to be several enzymes with multiple molecular forms. UK can exist as a single or two chain molecule (3) and as a fully active degraded form (4). Vascular activator, which is now more widely known as tissue-plasminogen activator (t-PA), can also exist as a single or two chain molecule (5) and it too, has been reported to have an active degraded form (6). In a normal system, the presence of different forms of these activators is highly possible and, based on potential different responses in different assays, can lead to very complex problems with interpretation of data (7).

Determination of PA activity levels can be further complicated by the presence of inhibitors of the activator and of the plasmin molecule generated by the activation of plasminogen. The use of direct assays employing amidolytic or esterolytic substrates can give a very different response than indirect assays based on the activation of plasminogen to plasmin. The subsequent measurement of plasmin from plasminogen can be complicated by binding differences between the enzymes in the reaction mixture.

The objective of this research was to better describe the plasminogen activators in conditioned medium (CM) from cultured cells, to demonstrate the need for multiple assays, and to detect the different

plasminogen activators and their multiple forms.

MATERIALS AND METHODS

Cells

Human kidney cells were received as frozen suspensions of primary cells from Whittaker M.A. Bioproducts (Walkersville, MD). The cells were cultured to confluence in flasks, harvested by trypsinization, suspended in a buffer, and separated based on their net surface charge by continuous flow electrophoresis as previously described (8). After separation, the cell subpopulations were collected and cultured to confluent density in growth medium containing 10% fetal calf serum. Two to ten cultures, depending on the number of cells separated, were set up for each subpopulation. At confluence, the cultures were changed to serum free maintenance medium containing lactalbumin hydrolysate and human serum albumin (9). Samples of this conditioned medium (CM) were removed at four or seven day intervals and stored at -70°C until assayed for PA activity. The data for replicate cultures from the individual subpopulations were averaged to determine the mean PA activity for each subpopulation.

Assays

FIBRIN PLATE LYSIS ASSAY (FPL): The standard assay described by Brakkman (10) and modified by the addition of plasminogen (11) was adapted as follows for use in this study. The following materials were placed in 15 X 100 mm disposable petri plates: 10 ml of 7.5 mg/ml

plasminogen free bovine fibrinogen (Miles Laboratories, Naperville, IL) in a 0.1 mol/l TRIS-HCl pH 8.0 buffer, 75 mg of human lys-plasminogen (American Diagnostica, Greenwich, CT), and 4 IU plasminogen free bovine thrombin (Miles Laboratories) in a 0.02 mol/l $CaCl_2$ - 0.15 mol/l NaCl solution. Clots were allowed to form for at least one hour at room temperature prior to applying CM samples. A 20 µl volume of each of four serial two-fold dilutions was applied to each of three plates per sample. After incubation for 18 hours at ambient temperature, the lysis zone diameters were read using a Transidyne General calibrating viewer (model 2743, Ann Arbor, MI). Zone diameters of the CM samples and their dilutions were compared to the standard (International Reference Preparation for urokinase, IRP-UK, England) for determination of UK activity.

MICRO-CLOT LYSIS ASSAY (MCLA): This assay, developed in our laboratory as described by Lewis et al. (12), utilizes 96 well flat bottom microwell plates. The reaction mixture in the wells consisted of a fibrinogen solution containing plasminogen, thrombin, and dilutions of the CM sample or IRP-UK standard. The optical density of the clots in each well was arbitrarily monitored at 405 nm at 5 minute intervals using an EIA automated microplate reader (Bio-Tek Instruments, Winoeski, VT, model EL310). Optical density data from the reader were transmitted via a serial interface directly to an Apple IIe® computer for which the software was written specifically to determine the assay endpoint (13). The endpoint, halflysis time, was defined as the time at which the clots in each well were half lysed as extrapolated from maximum and minimum optical density readings. As with the FPL assay, the activity for each

sample was derived by comparison of halflysis times to those of the IRP-UK standard.

AMIDOLYTIC ASSAY (S-2444): The amidolytic assay using the synthetic substrate Pyro-Glu-Gly-Arg-pNA (S-2444, KabiVitrum, Sweden) was essentially that described by Claeson et al. (14). For activation of latent PA in the conditioned medium, samples were incubated for 30 minutes at 37°C with human plasmin (American Diagnostica) at a final concentration of 2.3 µg/ml of sample volume. Activation was stopped by the addition of aprotinin (Trasylol ®, FBA Pharmaceuticals, New York, NY) at 10 KIU/ml in a TRIS-NaCl-HCl buffer at pH 8.8. S-2444 was then added to this solution in the wells of microtiter plates and the plates were incubated for an additional 4 hours at 37°C. The optical density was read at 405 nm using a Litton Bionetics (Charleston, SC) model LBI30 microplate reader. The direct assay plates (without plasmin incubation) were handled in the same manner except that an equivalent volume of the TRIS-NaCl-HCl buffer, instead of plasmin, was added to samples.

TISSUE PLASMINOGEN ACTIVATOR (t-PA): The materials and methods for the t-PA assay were contained in the IMUBIND ® t-PA ELISA kit obtained from American Diagnostica (15). The assay was modified to use medium containing human serum albumin as a diluent. The flat bottom microwell plates (Immulon 2 ®, Dynatech Laboratories, Alexandria, VA) were read at 490 nm on the EL310 autoreader.

PLASMIN INHIBITORS: To detect presence of plasmin inhibitors in CM, either the CM sample or unconditioned medium as a control was incubated with a known excess of human lys-plasmin. Residual plasmin activity was measured by the chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251,

KabiVitrum, Sweden) at 405 nm (16) using the LBI30 reader. Results were expressed as percent reduction in plasmin activity in the CM samples from the cultured cells compared to plasmin activity in the unconditioned medium.

Data analysis

All data from assays, whether they were zone diameters (FPL), optical density (S-2444 and t-PA), or halflysis time (MCLA), were analyzed by the parallel line method using natural log dose versus natural log response curves (13). Two-fold serial dilutions of the samples were compared with dilutions of the IRP-UK standard or the t-PA standard to determine relative activity in IU/ml for UK or concentration in ng/ml for t-PA.

RESULTS

PA ACTIVITY DETECTED BY FIBRINOLYTIC AND AMIDOLYTIC ASSAYS: A comparison of PA activity detected by the MCLA, FPL, and S-2444 assays is shown in Fig. 1 for CM from the human kidney cell subpopulations. The activity detected by the FPL assay was consistently higher than that detected by MCLA or S-2444. In the low mobility range, subpopulations 1-13, and high mobility range, 26-36, the activity detected by MCLA and S-2444 were comparable. In the conditioned medium from the midrange subpopulations, the PA activity detected by the MCLA was higher than that detected by S-2444. This could have been caused by the presence of a mixture of single chain urokinase-type plasminogen activator (scu-PA)

and the active double chain form (u-PA), since the plasmin generated in the activation of plasminogen by u-PA might, in turn, activate some scu-PA in the samples.

ACTIVATION OF scu-PA IN CONDITIONED MEDIUM SAMPLES: To determine if the differences between levels of activity detected by FPL, MCLA, and S-2444 assays were due to latent activator, CM samples were incubated with plasmin prior to S-2444 assay. As shown in Fig. 2, a 30 minute preincubation with plasmin resulted in an increase of up to 20 to 60 IU/ml for subpopulations 1-11 which had shown negligible activity in the direct S-2444 assay but significant PA levels by FPL. Thus, almost all of the PA in these subpopulations existed as scu-PA.

COMPARISON OF ASSAYS FOR DETECTING PLASMIN ACTIVATED PA: To determine the efficiency of each test for detecting activatable PA, a conditioned medium sample was divided into two aliquots. One aliquot was incubated for 1 hour at 37°C with plasmin (activated) and the other was incubated with buffer (direct). None of the direct tests detected all of the PA in the sample (Fig. 3). The MCLA and S-2444 showed approximately a 3-fold increase in activity and the FPL detected 1.5 times more activity after incubation of the sample with plasmin. That the sample activated with plasmin did not show as much activity in the FPL assay as detected by MCLA or S-2444 suggests that some, but not all, of the scu-PA converted to u-PA during the 18 hour direct FPL assay. However, the contribution to fibrinolysis of convertible PA is not defined by the FPL. Although the direct FPL consistently detected higher activity than S-2444 or MCLA direct assays (Fig. 1), the activated MCLA showed higher activity than the activated FPL or S-2444

for this typical sample. Thus, the MCLA appears to be useful for detecting highest levels of total plasmin activatable PA in CM samples.

PRESENCE OF PLASMIN INHIBITOR: The FPL and MCLA values for HK cell CM from another set of electrophoretically separated cells were very similar except for subpopulations in the range of 9-15 (Fig. 4A). In addition to scu-PA detected by S-2444 assay of plasmin activated samples, CM from subpopulations in the range of 9 to 16 (except 12) had significant antiplasmin activity (Fig. 4B) as detected by the S-2251 plasmin inhibitor assay described in "Materials and Methods". Samples outside this range showed virtually no inhibition. Thus, the presence of antiplasmin in these subpopulations appears to be an additional factor contributing to the differences between plasminogen activator activity as measured by the MCLA and FPL assays.

TOTAL PLASMINOGEN ACTIVATOR DETECTED BY THE BATTERY OF ASSAYS: The fibrinolytic, amidolytic, and an enzyme linked immunosorbent assay (ELISA) for total t-PA were performed on the CM from two to ten individual cultures for each separated subpopulation. Results, plotted as the average values (Fig. 5), show presence of active PA (MCLA, S-2444 direct, and FPL), scu-PA (calculable as the difference between direct and activated S-2444), and total t-PA in ng/ml (t-PA ELISA). Table 1 lists the type and range of linearity of each assay and the form of plasminogen activator measured. No assays for PA inhibitors were included in these studies because of sample volume limitations. Such assays should be a part of the test battery in order to better define the secretion by the cells of factors affecting the expression of plasminogen activation.

DISCUSSION & CONCLUSIONS

The primary conclusion from the research reported herein is that a battery of assays is required to define the various plasminogen activators in conditioned medium from cultured cells. Selected single assays do not accurately estimate the individual activators in the conditioned medium.

For example, while the FPL assay gives highest activity values, it does not define the contribution to fibrinolysis of double chain u-PA, t-PA, or the scu-PA converted to active u-PA during the assay. In addition, the FPL does not appear to be as sensitive as the other assays to the presence of plasmin inhibitor, possibly due to differences in diffusion coefficients between the 54 kd u-PA and higher molecular weight inhibitors. The finding that the FPL gives higher activity than the MCLA for samples containing scu-PA is not surprising. Since plasmin is formed during the fibrinolytic assays (17) and the FPL is a longer duration test (18 hours) compared to the MCLA (4 hours), this plasmin could in turn result in increased activation of the scu-PA which would then be detectable by the FPL. The smaller increase in PA activity detected by the FPL in the sample incubated with plasmin compared to the increase seen in MCLA and S-2444 assays (Fig. 4A) lends support to this conjecture.

The S-2444 direct assay preferentially detects active u-PA and does not detect scu-PA or fibrin dependent t-PA on an activity in IU/ml basis (18). In our studies, if samples had been tested only by direct S-2444, many of them would have been considered negative (Figs. 1 and 2). The

assay by S-2444 of samples incubated with plasmin detected u-PA in the sample and scu-PA converted to u-PA. Also, the activated S-2444 assay would not be affected by presence of antiplasmin, which would be oversaturated by the added plasmin. Thus, the combination of S-2444 plasmin activated and direct assays can be used to define the amount of scu-PA in the sample.

The MCLA is an automated, kinetic, fibrinolytic assay that is simple to perform (12). It detects u-PA, active t-PA, and an undetermined amount of scu-PA (converted in mixed samples containing u-PA and scu-PA in the absence of antiplasmin). The MCLA does not appear to detect scu-PA in CM unless plasmin is generated by u-PA present or the sample is preincubated with plasmin. After incubation of the CM with plasmin, the MCLA detects more PA activity than the activated FPL. This may be due to saturation of any antiplasmin present (Fig. 4B) and to single chain t-PA in the sample which could be converted to active double chain t-PA (19).

The rate of diffusion of the relatively large t-PA molecule (approximately 70 kd) in the FPL may affect the apparent activator activity. In mixtures of low and high molecular weight forms of PA's, the FPL assay may erroneously show only the contribution of the low molecular weight u-PA which has a higher diffusion rate. The high molecular weight forms, including t-PA and the 55 kd u-PA, diffuse outward more slowly than the lower molecular weight forms. Therefore, once the clot has already been lysed by the 34 kd u-PA, the activity of the larger PA's may not be measured in the lysis zone area of the FPL assay (20). If the incubation time is long enough, some lysis due to

the high molecular weight forms may occur. However, the activity of the large PA's cannot be accurately measured in the CM containing a mixture of both small and large PA enzymes. The size of the PA molecule is not a factor in either the classical fibrin clot lysis (tube) assay (21) or the MCLA. In these assays the reagents are mixed and distributed evenly throughout the clot system before lysis occurs so that the reaction becomes diffusion rate independent.

For a more complete characterization of PA, one should consider additional tests such as assays for inhibitors, inhibitor-activator complexes, and specific immunoassays. The results described in this report demonstrate that only by employing a battery of assays such as these described, can the true distribution of plasminogen activators in conditioned medium be established.

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. Paul Swank of KRUG International, Technology Life Sciences Division for the development of the computer software used in these assays.

This work was supported by NASA contract numbers NAS 9-17200 and NAS 9-16389.

REFERENCES

1. Williams J R B 1951 The fibrinolytic activity of urine. Br J Exp Pathol 32:530-537.

2. Aoki N, von Kaulla K N 1971 The extraction of vascular plasminogen activator from human cadavers and a description of some of its properties. Am J Clin Pathol 55:171-179.

3. Wun T-C, Ossowski L, Reich E 1982 A proenzyme form of human urokinase. J Biol Chem 257(12):7262-7268.

4. White W F, Barlow G H, Mozen M M 1966 The isolation and characterization of plasminogen activators (urokinase) from human urine. Biochemistry 5:2160-2169.

5. Wallén P, Rånby M, Bergsdorf N, Kok P 1981 Purification and characterization of tissue plasminogen activator: on the occurrence of two different forms and their enzymatic properties. In: Davidson J F et al. (eds), Progress in Fibrinolysis, Churchill Livingstone, Edinburgh, UK, 16-23.

6. Heussen C, Dowdle E B 1980 Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem 102(1):196-202.

7. Lewis M L, Morrison D R, Mieszkuc B J, Fessler D L 1984 Problems in the bioassay of products from HEK cells: Plasminogen Activator. In: Acton R T et al. (eds), Eukaryotic Cell Cultures, (Advances in Experimental Medicine and Biology; Vol 172), Plenum Press, New York, 241-267.

8. Lewis M L, Barlow G, Morrison D, Nachtwey D, Fessler D 1983 Plasminogen activator production by human kidney cells separated by continuous flow electrophoresis. In: Davidson J F et al. (eds), Progress in Fibrinolysis, Vol 6, Churchill Livingstone, Edinburgh, UK, 143-146.

9. Barlow G H, Rueter A, Tribby I 1975 Production of plasminogen activator by tissue culture techniques. In: Reich E et al. (eds), Proteases and Biological Control, Vol 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 325-331.

10. Brakkman K 1967 Fibrinolysis: a standardized fibrin plate method and a fibrinolytic assay of plasminogen. Scheltema and Holkema Publishing, Amsterdam.

11. Marsh N A, Gaffney P J 1977 The rapid fibrin plate - a method for plasminogen activator assay. Thromb Haemost 38:541-551.

12. Lewis M L, Nachtwey D S, Damron K L, Swank P R 1985 Development of a micro-clot lysis assay for urokinase. Thromb Haemost 54:102, Abstract P606.

13. Swank P R, Lewis M L, Damron K L 1986 Parallel line analysis: multifunctional software for the biomedical sciences. In preparation.

14. Claeson G, Aurell L, Friberger P, Gustavsson S, Karlsson G 1978 Designing of peptide substrates. Different approaches exemplified by new chromogenic substrates for kallikreins and urokinase. Haemostasis 7(2-3):62-68.

15. Bergsdorf N, Nilsson T, Wallén P 1983 An enzyme linked immunosorbent assay for determination of tissue plasminogen activator. Thromb Haemost 50:740-744.

16. Teger-Nilsson A C, Friberger P, Gyzander E 1977 Determination of a new rapid plasmin inhibitor in human blood by means of a plasmin specific tripeptide substrate. Scandinavian J Clin Lab Invest 37:403-409.

17. Lijnen H R, Zamarron C, Blaber M, Winkler M E, Collen D 1986 Activation of plasminogen by pro-urokinase (I. Mechanism). J Biol Chem **261**(3):1253-1258.

18. Rånby M, Norrman B, Wallén P 1982 A sensitive assay for tissue plasminogen activator. Thromb Res **27**:743-749.

19. Rånby M, Bergsdorf N, Nilsson T 1982 Enzymatic properties of the one- and two-chain form of tissue plasminogen activator. Thromb Res **27**:175-183.

20. Barlow G H, Francis C W, Marder V J 1981 On the conversion of high molecular weight urokinase to low molecular weight form by plasmin. Thromb Res **23**:541-547.

21. Plough J, Kjeldgaard N 0 1957 Urokinase: An activator of plasminogen from human urine. I: Isolation and properties. Biochim Biophys Acta **24**:278-282.

TABLE 1. PLASMINOGEN ACTIVATORS DETECTED BY EACH ASSAY

ASSAY	TYPE	RANGE	MEASURES
FPL	Fibrinolytic	2-20 IU/ml	u-PA Some scu-PA Active t-PA
MCLA (D)	Kinetic Fibrinolytic	2-200 IU/ml	u-PA Some scu-PA Active t-PA
MCLA (A)	Kinetic Fibrinolytic	2-200 IU/ml	u-PA scu-PA converted to u-PA Active t-PA
S-2444 (D)	Amidolytic	2-20 IU/ml	u-PA
S-2444 (A)	Amidolytic	2-20 IU/ml	u-PA scu-PA converted to u-PA
t-PA	ELISA	1-16 ng t-PA/ml	Total t-PA

LEGENDS

Fig. 1 Plasminogen activator activity in conditioned medium from human kidney (HK) cell subpopulations as detected by amidolytic S-2444 (□), micro-clot lysis (+), and fibrin plate lysis (◇) assays.

Fig. 2 Amidolytic activity detected by S-2444 assay after incubation of conditioned medium samples with plasmin for 30 minutes (activated, △) compared to activity of the same samples not incubated with plasmin (direct, □).

Fig. 3 Comparison of the micro-clot lysis, fibrin plate lysis, and amidolytic S-2444 assays for detection of plasminogen activator in a typical conditioned medium sample from human kidney cells. The sample was incubated with either plasmin (Activated, **A**) or with plasmin-free buffer (Direct, **D**).

Fig. 4A Comparison of the fibrin plate (+) and micro-clot lysis assays (□) for detecting fibrinolytic activity in conditioned medium from HK cell subpopulations. Both assays showed comparable activity except for the subpopulations in the range 9-15.

Fig. 4B Presence of plasmin inhibitor in conditioned medium was demonstrated by detection of the residual plasmin activity by S-2251 after addition of excess lys-plasmin to the samples. Plasmin inhibitor is expressed as a percent of the activity in conditioned compared to unconditioned medium.

Fig. 5 Plasminogen activators detected by a battery of five assays. These plots show the presence of active u-PA, (micro-clot lysis (+), S-2444 direct (□), and fibrin plate lysis (△) assays), scu-PA (calculable as the difference between S-2444 direct (□) and S-2444 activated (◇), and total t-PA in ng/ml (enzyme linked immunosorbent assay) (x).

FIG 1

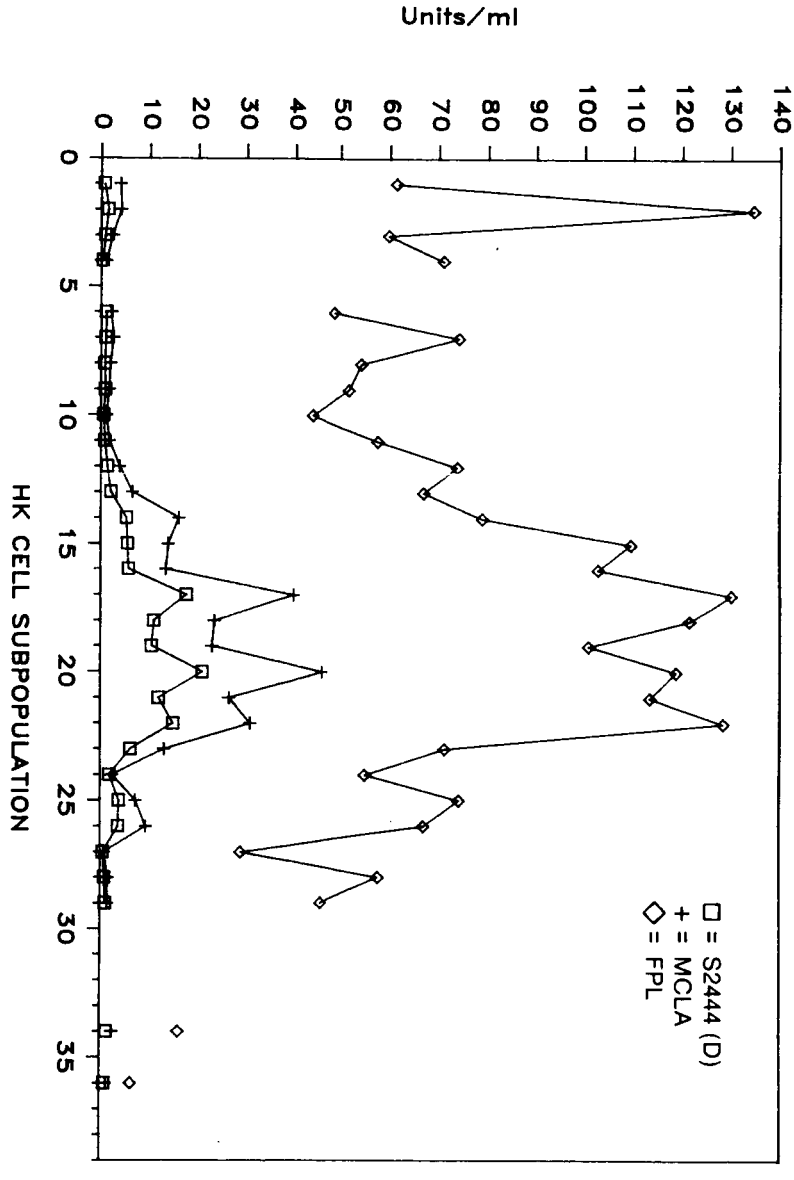

Units/ml
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
HK CELL SUBPOPULATION
□ = S2444 (D)
+ = MCLA
◇ = FPL

FIG 2

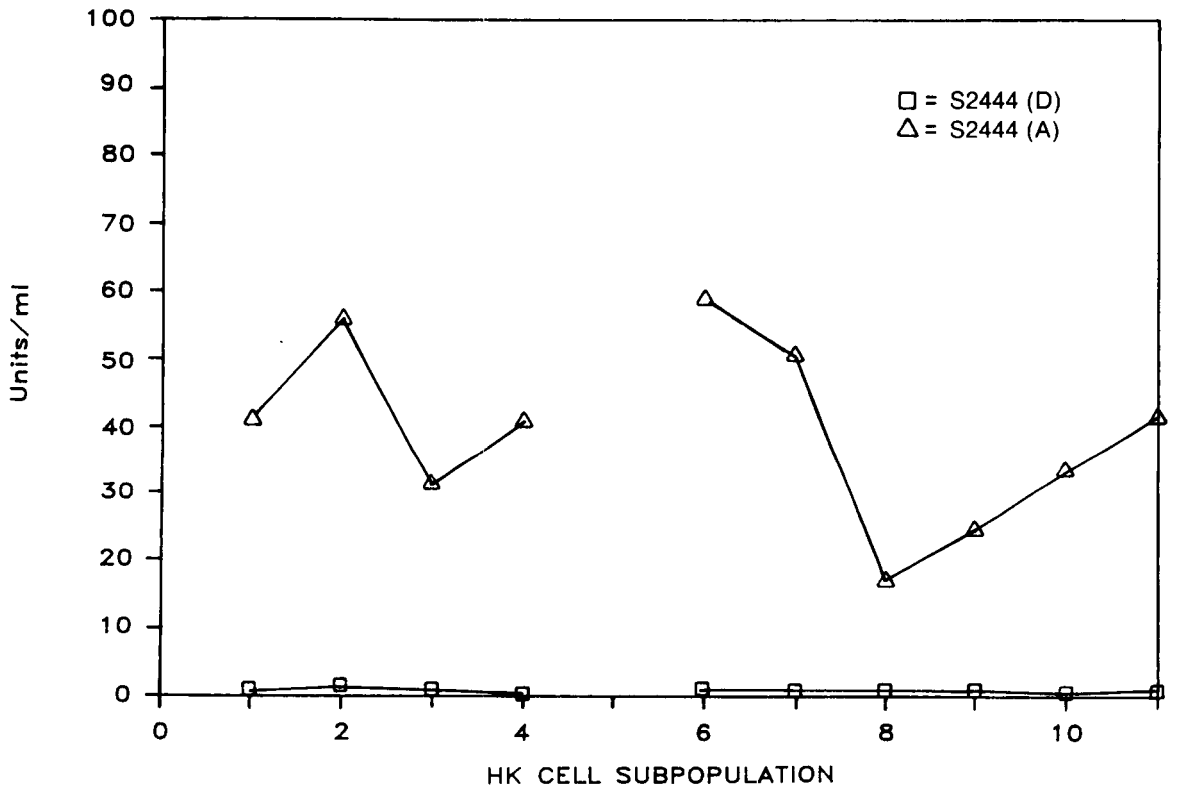

100
90
80
70
60
50
40
30
20
10
0
Units/ml
□ = S2444 (D)
△ = S2444 (A)
0
2
4
6
8
10
HK CELL SUBPOPULATION

FIG 3

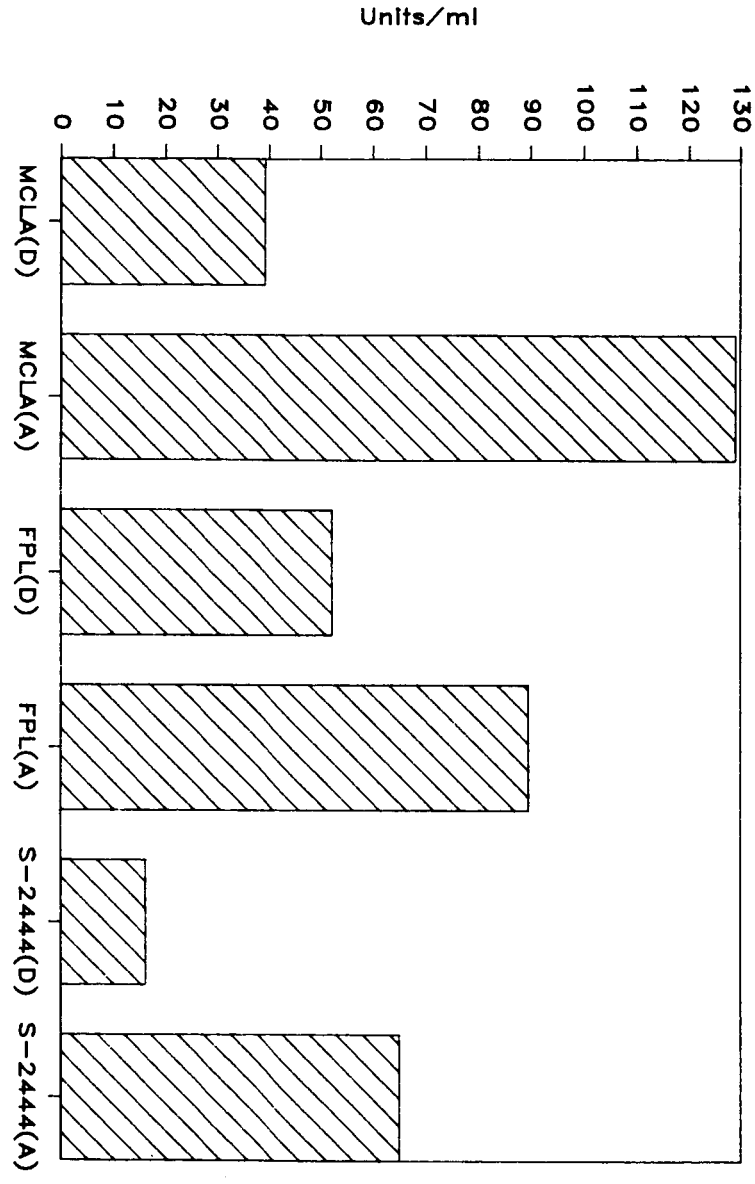

Units/ml
0
10
20
30
40
50
60
70
80
90
100
110
120
130
MCLA(D)
MCLA(A)
FPL(D)
FPL(A)
S-2444(D)
S-2444(A)

FIG 4A

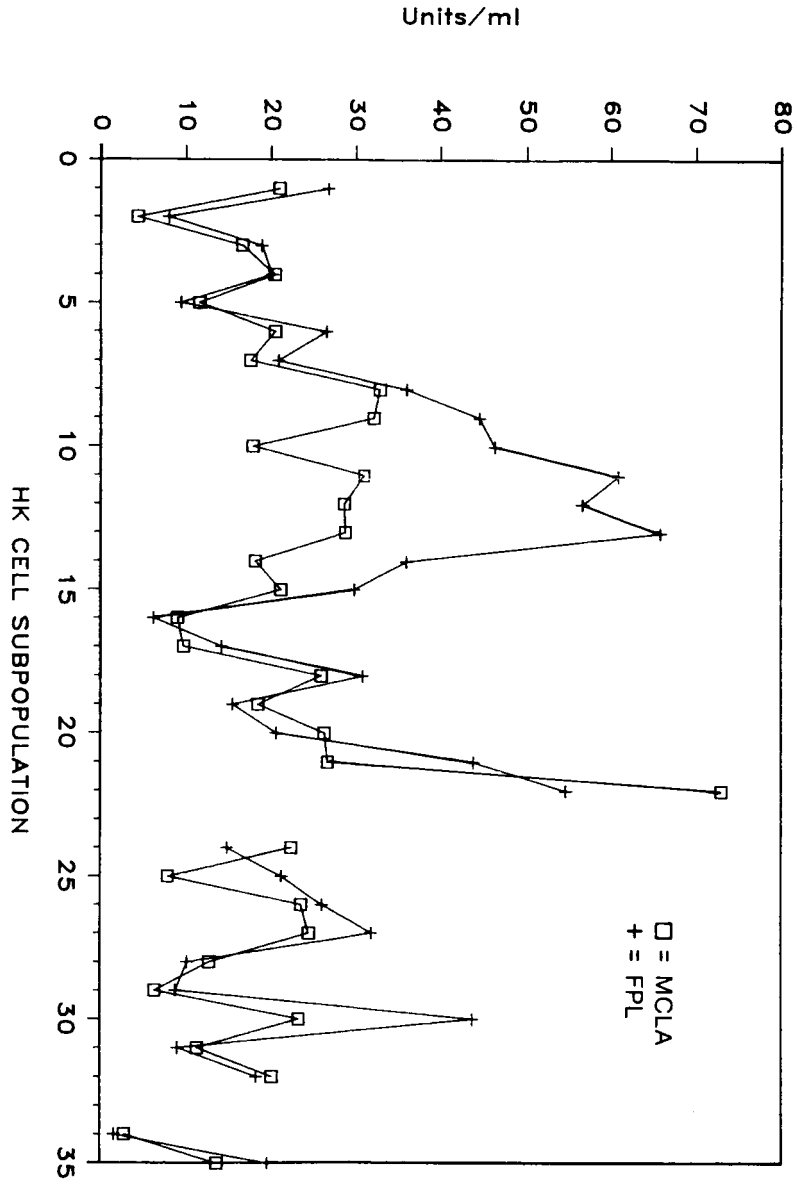

Units/ml
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
HK CELL SUBPOPULATION
□ = MCLA
+ = FPL

FIG 4B

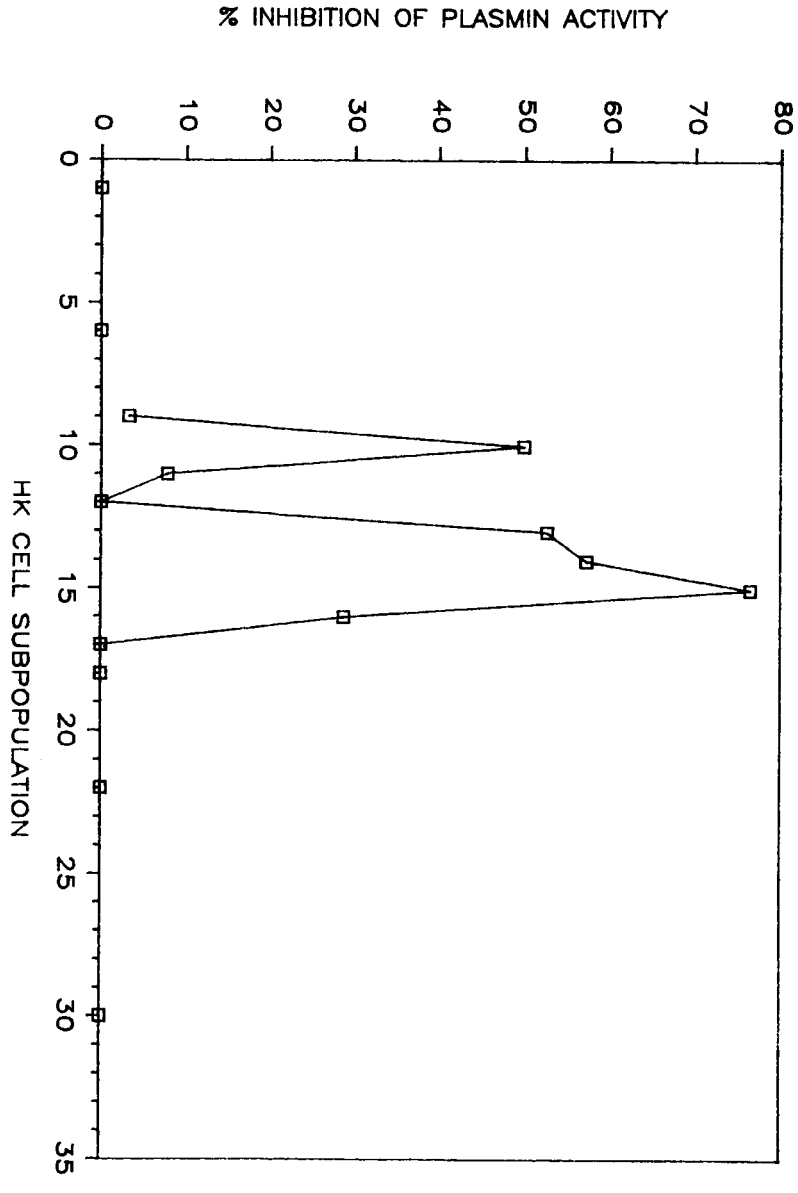
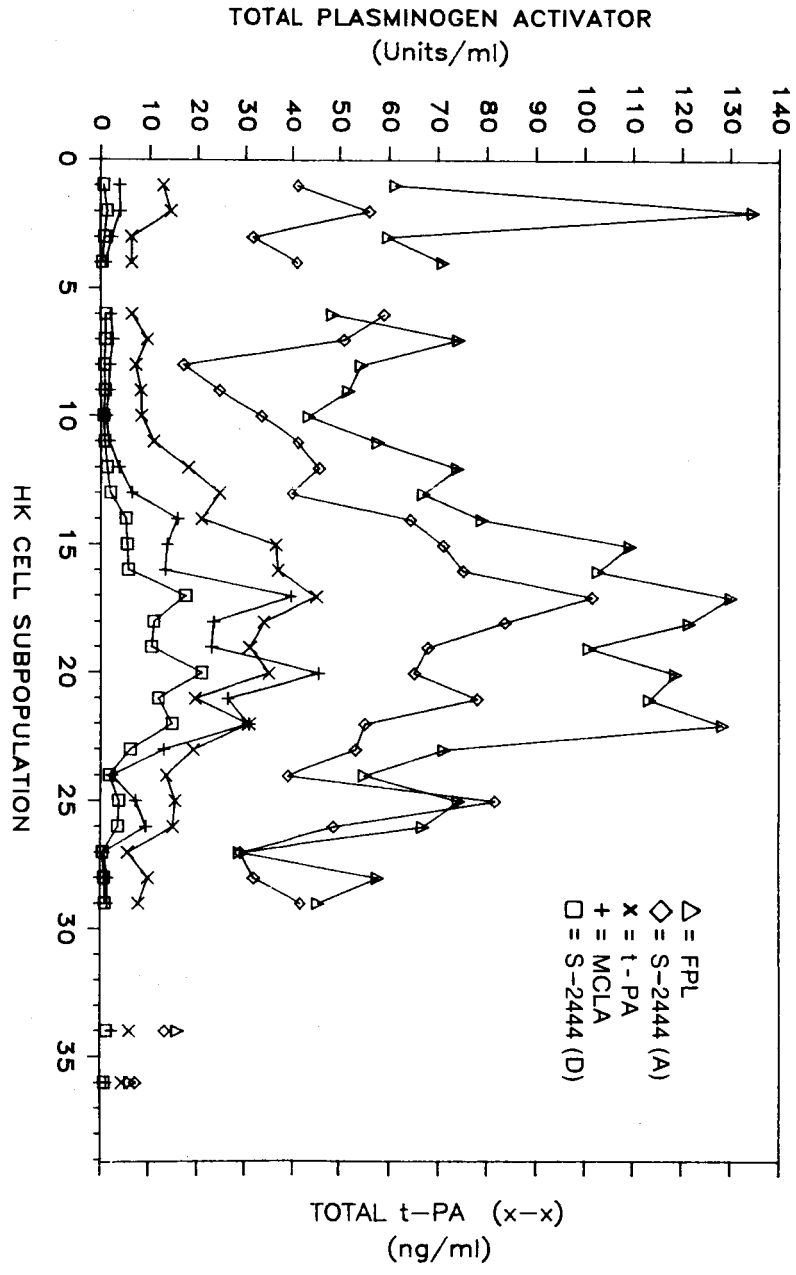

% INHIBITION OF PLASMIN ACTIVITY
0
10
20
30
40
50
60
70
80
HK CELL SUBPOPULATION
0
5
10
15
20
25
30
35

Fig 5


TOTAL PLASMINOGEN ACTIVATOR
(Units/ml)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
HK CELL SUBPOPULATION
0
5
10
15
20
25
30
35
△ = FPL
◇ = S-2444 (A)
× = t-PA
+ = MCLA
□ = S-2444 (D)
TOTAL t-PA (x-x)
(ng/ml)